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Compartmentation of Newly Synthesized Phosphatidylethanolamine in Rat Brain Microsomes

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Summary. The compartmentation of the phosphatidylethanolamine newly synthesized in brain microsomes *in vitro* either by base exchange or net synthesis has been studied, using difluorodinitrobenzene as a chemical probe. The experimental results demonstrate that in rat brain microsomes the phosphatidylethanolamine molecules synthesized by base exchange and the bulk membrane lipid belong to different pools. Ca^{2+} bound to microsomes seems to be involved in the maintenance of the compartmentation of phosphatidylethanolamine. In the presence of Ca^{2+} the newly synthesized phosphatidylethanolamine molecules react with difluorodinitrobenzene as though they are organized in clusters. After biosynthesis *in vivo* or *in vitro* through the cytidine pathway, the compartmentation of the newly formed phosphatidylethanolamine appears less marked than after the synthesis through base exchange.

Key Words phosphatidylethanolamine · cross-linking · brain microsomes · divalent cations

Introduction

The concept that protein and lipid mobility in membranes is not simply regulated by the rules of diffusion is generally accepted. Indeed, many factors seem to be involved in the control of membrane dynamics and of its nonrandom organization [19].

It has been shown in the last decade that lipids are asymmetrically distributed between the two leaflets of some natural membranes [37]. Moreover, the uneven organization of membrane lipids does not seem to be restricted to transverse asymmetry, since a heterogeneous distribution of lipids on the lateral plane of the membranes has also been reported [15, 28]. Indeed, there is evidence supporting membrane models in which lipids are organized in domains [4, 5, 8, 27, 33, 39, 40, 42] and this could have important functional implications.

Also the membranes of the endoplasmic reticulum, which contain most lipid-metabolizing enzymes, exhibit an asymmetric phospholipid distribution [17, 35]. Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are synthesized in the endoplasmic reticulum by several pathways, among which are the base-exchange reaction and the cytidine pathway [1, 26, 29]. The latter mechanism operates the conversion of apolar membrane components into amphiphilic lipid molecules, which should imply a large perturbation of the membrane because of the different interfacial characteristics of the precursor (diglyceride) and the product (phospholipid). This perturbation could provide the driving force for lateral and transverse movements of lipids [11, 14]. Similar speculations can be made for the phospholipid molecules synthesized by base exchange.

It has been demonstrated that the PE and the PC synthesized both in liver and brain microsomes from labeled precursors are firstly located only on the cytoplasmic side of the vesicles and later diffuse to the luminal surface [6, 7, 18, 21, 23, 24].

In the present paper, evidence suggesting the clustering of the PE molecules synthesized through base exchange in rat brain microsomes is reported.

Materials and Methods

[1-^{3}H]ethanolamine hydrochloride (specific activity, 19.5 Ci/mmol) and CDP-[1,2-^{14}C]ethanolamine (specific activity, 97 Ci/mol) were purchased from Amersham International (U.K.). 1,5-difluoro-2,4-dinitrobenzene (DFDNB) was obtained from Pierce Eurochemie (The Netherlands). Other chemicals were purchased from Carlo Erba (Italy). Organic solvents were freshly distilled before use.

Cross-Linking Between PE Molecules Labeled In Vitro

Microsomes were isolated from adult rat brain and characterized as previously described [38]. Isolated microsomes (30 mg protein) were incubated 30 min at 30°C with 20 μCi of CDP-[1,2-^{14}C] ethanolamine (specific activity, 97 Ci/mol) in the pres-

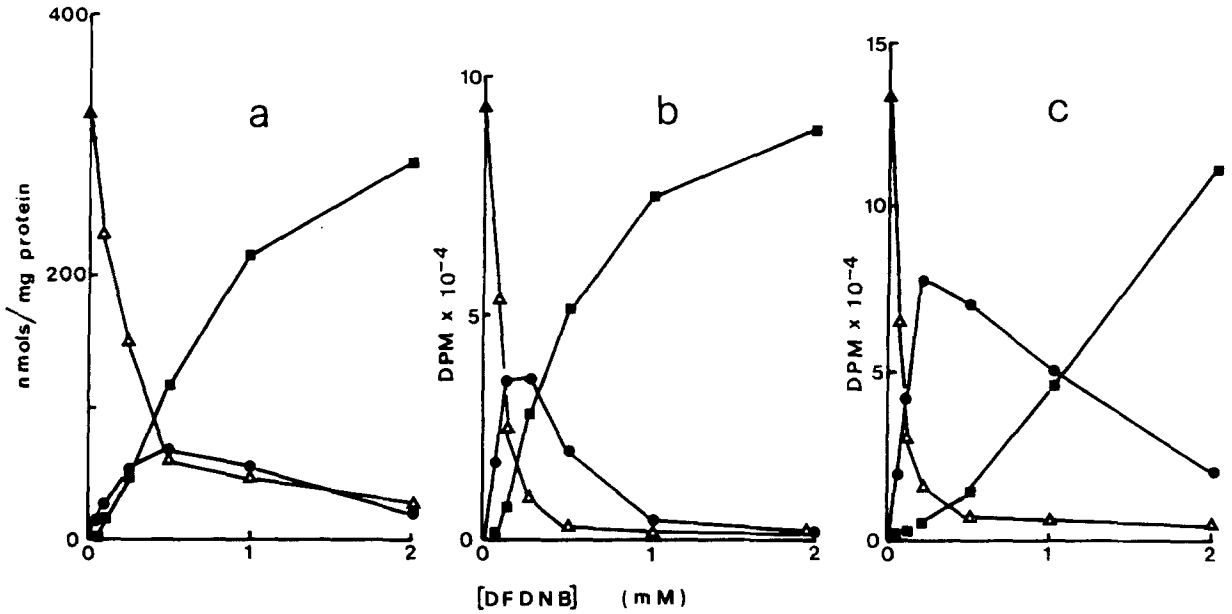

Fig. 1. Reaction of microsomal PE with DFDNB. *a*) Microsomes (1.5 mg protein) were reacted for 2 hr at 20°C with 3 ml of 0.16 M $NaHCO_3$ containing DFDNB. *b,c*) After labeling PE through net synthesis (*b*) or by base exchange (*c*), microsomes were pelleted and resuspended in sucrose. Aliquots of the suspensions (*b*, 1 mg protein; *c*, 0.4 mg protein) were reacted with DFDNB as in *a*. Data are expressed as nmol/mg protein (*a*) or DPM (*b* and *c*) and represent the average values from three separate experiments. Standard deviation was less than 10%. (●---●---●), PE-DNPh-PE; (■---■---■), FDNPh-PE; (△---△---△), unreacted PE

ence of 10 mM $MnCl_2$, 40 mM HEPES buffer (pH 8.0) and 0.2 M sucrose in a final volume of 8 ml.

In other experiments the microsomal PE was labeled through the base-exchange reaction incubating 30 min at 30°C the microsomes (30 mg protein) with 10 μCi of [1-^{3}H]ethanolamine (specific activity, 19.5 Ci/mmol), 40 mM HEPES buffer (pH 8.0), 2.5 mM $CaCl_2$, in a final volume of 8 ml.

The incubations were stopped by cooling the mixtures to 0°C. The labeled microsomal suspensions were diluted to 35 ml with 0.32 M sucrose and centrifuged 1 hr at 104,000 × *g* at 4°C. The microsomal pellets were suspended in 10 ml of 0.32 M sucrose. Aliquots of these suspensions, containing about 1 mg microsomal protein, were reacted with two volumes of a solution containing 0.16 M $NaHCO_3$ and 75 μM to 3 mM DFDNB. The mixtures were kept for 2 hr at 20°C and the lipids extracted and isolated by TLC [42].

The cross-linked PE (PE-DNPh-PE) and the monoderivatized PE (FDNPh-PE) were identified according to Baumgarten et al. [2], after acid hydrolysis [34]. PE-DNPh-PE and FDNPh-PE were eluted from the silica with chloroform/methanol (2:1, by vol) and determined spectrophotometrically at 333 nm [2]. The same samples were then transferred to scintillation vials, the solvent was evaporated and the radioactivity measured by liquid scintillation. Unreacted PE was determined as P_i. Aliquots were counted for radioactivity.

Cross-Linking Between PE Molecules Labeled In Vivo

[1-^{3}H]ethanolamine (30 μCi, specific activity, 19.5 Ci/mmol) was injected intraventricularly into rat brain. Brains were excised after 1 or 24 hr and the microsomal fraction was isolated [38]. Microsomes were reacted with DFDNB; and PE-DNPh-PE, FDNPh-PE and underivatized PE were isolated and quantitated as described above.

Removal of Divalent Cations from Microsomes

After labeling the microsomal PE by base exchange or by net synthesis, microsomes were pelleted and resuspended in 0.32 M sucrose (5 mg protein/ml). The microsomal suspensions were mixed with the same volume of an isotonic solution containing 6 mM EDTA and incubated at 30°C for 2 hr. Controls were incubated with 0.32 M sucrose. After incubation, the membranes were pelleted at 104,000 × *g* for 1 hr and the membrane-bound Ca^{2+} or Mn^{2+} were quantitated.

The amount of Ca^{2+} associated to microsomes was determined according to Diehl and Ellingboe [16]. The membrane-bound Mn^{2+} was quantitated colorimetrically at 545 nm after the mineralization of microsomes with perchloric acid and oxidation of manganese to permanganate with periodic acid [44].

Cross-Linking Between PE Molecules after Removal of the Membrane-Bound Cations

Membrane PE was labeled *in vitro* by base exchange or by net synthesis. Labeled microsomes were pelleted and resuspended in 0.32 M sucrose (5 mg protein/ml). 0.25 ml of the microsomal suspensions were mixed with 0.25 ml of 6 mM EDTA in 0.32 M sucrose and kept 2 hr at 30°C. 1 ml of 0.06 to 2.5 mM DFDNB in 0.16 M $NaHCO_3$ was added to the incubation mixture and the reaction was carried out for 2 hr at 20°C. Controls were treated in the same way, but, after incubation with radioactive precursors, microsomes were incubated with a sucrose solution not contain-

Table 1. Specific radioactivity of PE and PE derivatives after reaction with DFDNB of microsomes labeled *in vivo* or *in vitro*, and effect of EDTA

		PE-DNPh-PE	FDNPh-PE	Unreacted PE
[a]base exchange	*a*)	317	794	141
	b)	201	356	264
[a]net synthesis	*a*)	332	809	147
	b)	324	506	382
[b]*in vivo*	1 hr)	109	154	42
	24 hr)	135	142	160

[a] The microsomal PE was labeled through net synthesis or through base-exchange reaction. *a*) Control microsomes were reacted with 0.25 mM DFDNB. *b*) Microsomes were incubated 2 hr at 30°C with 3 mM EDTA, before the reaction with 0.25 mM DFDNB.

[b] The microsomes were isolated from rat brain after 1 or 24 hr from the intracerebroventricular injection of radioactive ethanolamine, then reacted with 0.25 mM DFDNB. PE-DNPh-PE, FDNPh-PE and unreacted PE were isolated and their specific activity measured. Specific activity values are expressed in DPM/nmol of lipid phosphorus. Data represent the average values from three experiments. Standard deviation was less than 10%.

ing EDTA. The labeled lipids were isolated and quantitated as described above.

Analyses

Lipid phosphorus was determined by the method of Ernster et al., after mineralization [20]. Protein was evaluated according to Lowry et al. [30]. Radioactivity was measured with a 460-CD Tri-Carb counter (Packard Instrument Co., USA), with Emulsifier Packard® as liquid scintillator.

Results

Cross-Linking Between PE Molecules Labeled In Vitro

The reaction of microsomes with DFDNB produced an extensive cross-linking between PE molecules, the maximum dimer formation being reached when microsomes were reacted with 0.5 mM DFDNB (Fig. 1*a*). At higher DFDNB concentrations, the amount of the dimer formed during reaction decreased, whereas a concomitant increase of FDNPh-PE was obtained.

Part of the membrane PE was cross-linked with proteins, the maximum of PE-DNPh-protein formation being reached at about 0.5 mM DFDNB (about 5% of the whole membrane PE).

Net Synthesis

The microsomal PE molecules newly synthesized through the Kennedy pathway did not follow the same derivatization pattern of the unlabeled bulk PE. About 30% of the labeled PE was not recovered in the lipid fraction after derivatization with 0.2 mM DFDNB and was bound to protein. As shown in Fig. 1*b*, the radioactivity of PE-DNPh-PE reached a maximum in the same conditions. Moreover, after the reaction of the microsomes with 1 mM DFDNB, more than 90% of the radioactive molecules of PE were associated with FDNPh-PE.

As a consequence of the different derivatization pattern of radioactive and bulk PE molecules, the specific activity values of PE-DNPh-PE, FDNPh-PE and PE were different (Table 1). In particular, after reaction with 0.25 mM DFDNB, the specific activities of PE-DNPh-PE and FDNPh-PE were higher than that of the unreacted PE, indicating that the probe reacted preferentially with the radioactive PE molecules.

About 0.35 μmol of Mn^{2+} per μmol of phospholipid were bound to the microsomes incubated in the conditions for the net synthesis. Following EDTA treatment, the membrane-bound Mn^{2+} was reduced almost to zero and the specific activity of derivatized and underivatized PE became closer (Table 1).

Base Exchange

Microsomal PE labeled *in vitro* by base exchange was reacted with DFDNB and the radioactivity of PE-DNPh-PE, FDNPh-PE and unreacted PE were plotted versus DFDNB concentration (Fig. 1*c*). The derivatization pattern of the PE labeled by base exchange differs greatly from that labeled by net synthesis. Although the maximum cross-linking between labeled PE molecules was reached at the same DFDNB concentration, a larger percentage of the radioactive PE was found as PE-DNPh-PE, after labeling through base exchange. At 1 mM

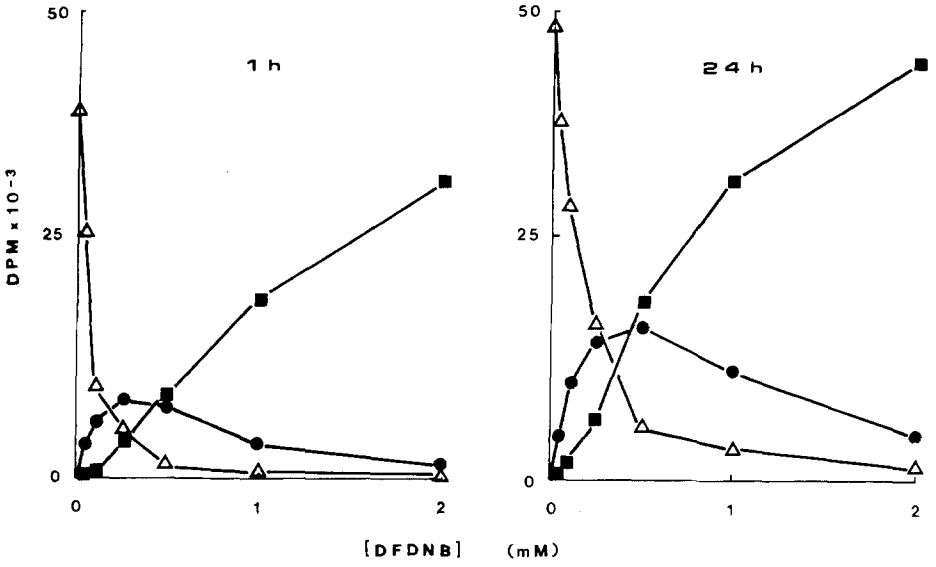

Fig. 2. Cross-linking of the microsomal PE labeled in vivo. Rat brain microsomal PE was labeled with 30 μCi of [1-^{3}H] ethanolamine injected intracerebroventricularly. Microsomes were isolated after 1 and 24 hr and reacted with DFDNB (1.5 mg protein/ml). Data are expressed as DPM of PE-DNPh-PE (●---●---●), FDNPh-PE (■---■---■) and unreacted PE (△---△---△) isolated from microsomes containing 3 mg protein. Triplicate experiments gave the same labeling pattern shown in the figure

DFDNB concentration, the amount of radioactivity associated with PE-DNPh-PE was comparable to that associated with FDNPh-PE. About 25% of the labeled PE was not recovered in the lipid fraction after reaction of microsomes with 0.2 mM DFDNB and was bound to protein.

The specific activity of PE-DNPh-PE and of FDNPh-PE was higher than that of the unreacted molecules of PE, indicating that the newly synthesized molecules reacted preferentially with the probe (Table 1). After incubation of microsomes in a standard medium for the base-exchange reaction, about 0.12 μmol of Ca^{2+} per μmol of phospholipid were bound to microsomes. This amount decreased virtually to zero after treatment with EDTA. The incubation of the labeled microsomes with 3 mM EDTA, before the reaction with DFDNB, produced the same effects described for microsomes labeled by net synthesis, namely, the specific activities of the different PE pools became closer (Table 1).

Cross-Linking Between PE Molecules Labeled In Vivo

The reaction with DFDNB was carried out on microsomes whose PE had been labeled *in vivo* by [1-^{3}H]ethanolamine injected intracerebroventricularly. After 1 and 24 hr from the isotope injection, the microsomes were isolated and exposed to DFDNB. The reaction of radioactive PE with DFDNB exhibited little difference between 1 and 24 hr. The maximal cross-linking between radioactive PE molecules was obtained with 0.25 and 0.5 mM DFDNB, after 1 and 24 hr, respectively (Fig. 2).

An extensive cross-linking of labeled PE with proteins occurred in the 1-hr experiments (*results not shown*) and, therefore, at 0.1 mM DFDNB concentration, the decrease of the radioactivity of unreacted PE molecules was accompanied by a minor increase of the radioactivity in FDNPh-PE and PE-DNPh-PE. This was much less evident in the 24-hr experiments.

The specific activities of PE and PE derivatives are reported in Table 1. The specific activities of PE-DNPh-PE and FDNPh-PE formed during incubation of microsomes with 0.25 mM DFDNB was higher than that of the unreacted PE, in the 1-hr experiments. On the contrary, PE and PE derivatives had similar specific activities when the microsomes were isolated 24 hr after the isotope injection.

Dimer/Monomer Ratio

The reaction of the membrane PE with DFDNB led to the formation of monomer and dimer derivatives (FDNPh-PE and PE-DNPh-PE, respectively). The dimer/monomer ratio, calculated at very little DFDNB concentrations, gives an indication of the average distance between the PE molecules in the membrane [31, 32]. This ratio has been calculated both for the radioactivity and for the mass of dimer and monomer formed in the presence of 0.1 mM DFDNB (Table 2).

The microsomal PE labeled *in vitro* by base exchange was efficiently cross-linked in the presence of 0.1 mM DFDNB. In fact, the dimer/monomer radioactivity ratio was much higher than the dimer/monomer molar ratio. Radioactivity ratio and molar ratio became comparable at higher DFDNB concen-

trations or by preincubating labeled microsomes with EDTA before the reaction with the cross-linker. The labeling ratio was higher than the molar ratio also in the microsomes labeled *in vitro* by net synthesis and a significant decrease was observed when microsomes were preincubated with EDTA. Also the labeling ratio was higher than the molar ratio in the microsomes labeled *in vivo*.

Discussion

In previous studies it has been demonstrated that:

1. PE and PC are synthesized on the cytoplasmic leaflet of the rat brain endoplasmic reticulum [3, 6, 9, 18].
2. The PE molecules synthesized *in vitro* from CDP-ethanolamine through net synthesis equilibrate between the two faces of the microsomal vesicles [18], the rate of the equilibration process being related to the binding of divalent cations to the membranes [3].
3. A very efficient equilibration of the newly synthesized PE between the two membrane leaflets occurs after its biosynthesis *in vivo* [3, 6].

In the present work, the membrane distribution of the radioactive PE molecules synthesized *in vivo* and *in vitro* has been examined using DFDNB as a probe. DFDNB can produce cross-linking between two adjacent PE molecules, thus providing information concerning the distribution of the newly formed radioactive PE molecules on the lateral plane of the membrane [31, 32].

The results obtained indicate that the PE molecules newly synthesized *in vitro* are better accessible to DFDNB than the bulk microsomal PE. In fact, the specific activity of the PE derivatized with DFDNB was higher than that of the unreacted PE (Table 1). Since the PE molecules newly formed *in vitro* are mainly exposed on the outer leaflet of microsomes, immediately after synthesis [3], uneven specific activities of PE and PE derivatives suggest that DFDNB reacts preferentially with the PE molecules exposed on the outer leaflet of the microsomal vesicles. Indeed, although the lipid bilayer is virtually permeable to DFDNB, the high reactivity of the probe might result in preliminary titration of the primary amino groups of the outer leaflet, followed by the reaction with the internal PE molecules. This could explain why, increasing the DFDNB concentration, the specific activity of the PE derivatives decreases.

Concerning the availability of the membrane PE for the formation of dimers the present results indicate that the radioactive PE molecules synthesized through base exchange are more available for the formation of dimers than those synthesized *in vivo* or through the cytidine pathway (Table 2). Moreover, after labeling PE through base exchange, the dimer/monomer labeling ratio is about ten times higher than the molar ratio. A dimer/monomer radioactivity ratio greater than twice the molar ratio indicates that the newly formed PE molecules are organized in clusters on the plane of the membranes. This is the case of the microsomal PE labeled through base exchange.

Table 2. Dimer/monomer labeling and molar ratios calculated after labeling microsomal PE either *in vivo* or *in vitro*

Molar ratio	1.7 ± 0.2
Labeling ratio	
a) base exchange[a]	18.8 ± 2.1
b) net synthesis[a]	5.3 ± 0.8
c) base exchange/EDTA[b]	2.6 ± 0.7
d) net synthesis/EDTA[b]	3.2 ± 0.6
e) in vivo (1 hr)[c]	4.9 ± 0.6
f) in vivo (24 hr)[c]	5.4 ± 0.6

Dimer/monomer ratio was calculated after the reaction of microsomes with 1×10^{-4} M DFDNB. Dimer/monomer labeling ratio was calculated by dividing the radioactivity of PE-DNPh-PE by that of FDNPh-PE. The dimer/monomer molar ratio was calculated as mols of PE-DNPh-PE/mols of FDNPh-PE. Data represent the average of three experiments at least, ±SEM.

[a] Microsomal PE was labeled through base exchange (*a*) or net synthesis (*b*) before the reaction with DFDNB, as described under Materials and Methods.

[b] After labeling microsomal PE through base exchange (*c*) or net synthesis (*d*), microsomes were pelleted, resuspended and incubated at 30°C for 1 hr in isotonic sucrose containing 3 mM EDTA.

[c] Microsomes were prepared 1 hr (*e*) or 24 hr (*f*) after the intracerebral injection of tritiated ethanolamine to rats.

The incubation with EDTA of microsomes labeled through base exchange decreases the amount of radioactive PE available for the formation of dimers. This suggests that the labeled PE molecules and the whole membrane lipid equilibrate when the membrane-bound Ca^{2+} ions are removed.

Although any further speculation on the present data is weakened by the great heterogeneity of the brain microsomal vesicles, the role that the membrane-bound cations play on the cross-linking between PE molecules is evident.

The ion effect put into evidence in the present paper can be related to previous observations [10, 12, 13, 22, 25, 36, 41, 43] demonstrating that the assembly of both natural and artificial membranes strongly depends on their ionic environment.

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